CARBON DIOXIDE AS A NARCOTIC AGENT.

I. The Effect of Carbon Dioxide upon the Fertilized Egg of *Arbacia*.

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The narcosis which may be produced by carbon dioxide seems to have long been known. Pliny the Elder, in his Natural History, remarked that the marble from Memphis, when ground up and used as a liniment with vinegar, had the virtue of rendering insensible parts of the body to be cut or cauterized. In modern times the value of carbon dioxide as an analgesic was recognized soon after Black's discovery of "fixed air," and in 1788 we find Percival recommending "fixed air" for the relief of painful ulcers. Later it was employed as a local anesthetic by Ingenhousz,[1] Beddoes, Simpson, Follin, Brown-Séquard,[2] Gellé,[2] and others. In 1828, eighteen years before Morton's demonstration of ether anesthesia, Hickman[3] appears to have suggested that general anesthesia be induced by inhalations of carbon dioxide, and it was thus used later in the century by Ozanam and by Gréhant, although never extensively. Ozanam seems to have been impressed by the promptness of recovery from the anesthesia produced in this way. At one time it was thought by some that natural sleep and hibernation were the effects of the accumulation of carbon dioxide. This view has been abandoned, but Kidd (1914) has recently with more plausibility suggested a similar explanation for the dormancy of seeds. The work of Cohn (1918) also indicates the possibility that carbon dioxide may play an important part in keeping the spermatozoa of *Arbacia* inactive until their discharge into sea water.

[1] Cited by Herpin (1864).
[2] Cited by Dastre (1890).
[3] Cited by Simpson (1856).

Inasmuch as carbon dioxide is a normal product of metabolism, an understanding of its characteristic effects on living cells and the mechanism by which they are produced is of importance. Unfortunately, most of the studies previously made on its action have been concerned with entire organisms where numerous complicating factors prevent the separation of its more characteristic effects from others of a less fundamental nature. It is the object of the present paper to study in a quantitative way certain aspects of the narcotic effects of carbon dioxide on single cells—the developing egg cells of *Arbacia*—where complicating factors are reduced to a minimum. In particular, an attempt has been made to determine the extent to which such effects are reversible. A second paper [1] deals similarly with a simple tissue—the striated muscle of the frog—and studies upon ciliated epithelium are to be reported elsewhere.

For quantitative work, the developing egg of *Arbacia* proves very satisfactory. Since favorable material shows about 100 per cent. division under normal conditions, any delay in the occurrence of the first cleavage may be measured and used as a suitable quantitative criterion for narcotic or toxic effects upon the activity of the cell. Smith and Clowes (1924) have studied from a somewhat different point of view from that of the present paper the effects of carbon dioxide upon the cleavage of echinoderm eggs and have found that development is inhibited by CO_2 at pH values which in its absence are without effect upon cleavage. The present experiments differ in at least two respects from those of Smith and Clowes. In the first place, they have to do with exposures of varying lengths *followed by a return to normal sea water* to permit a determination of the extent to which the effects produced are reversible. In the second place, a different method has been used for the quantitative measurement of the degree of retardation of the developmental processes. Instead of measuring the total number of cell divisions secured in a given time irrespective of whether they be the first, second, or third, the quantity here measured has been the time required for the first cleavage in each case. Furthermore, this has been done in such a way as to take into account

[1] *Amer. Journ. Physiol.*, LXXXII., 241.

not merely the mean time for all of the eggs but, approximately at least, the time for each individual egg. It is believed that data of this sort, while more troublesome to secure, are theoretically more significant and have a wider range of usefulness than those obtained by the other method.

Method.

Sea water, saturated with CO_2 from a Kipp generator, was used directly or was diluted as desired with oxygen-saturated sea water, with nitrogen-saturated sea water, or with ordinary sea water by siphoning together appropriate amounts of these solutions. It was then immediately siphoned into 75 cc. glass-stoppered bottles containing a few drops of a concentrated suspension of the newly fertilized eggs of *Arbacia punctulata*. As soon as the bottles were completely filled, they were tightly stoppered, shaken briefly, and placed in a bath of running sea water, which, with a few exceptions, varied in temperature not more than 0.5° C. for the duration of each experiment. For all the experiments during one season the range of temperature was 18.4° C. to 22.4° C., with a mean value of 20.3° C. In order to prevent a change in the solubility of the dissolved gases, it was deemed important always to have the CO_2-containing solutions at the temperature of the water-bath. Care was also taken to begin all the exposures of any one series as nearly simultaneously as possible, since a number of experiments not reported here seemed to indicate that sensitiveness to CO_2 may vary prior to the appearance of the first cleavage. In many of the experiments the bottles were inverted at five minute intervals to keep the contents well mixed, but other experiments in which this precaution was less rigorously observed gave essentially the same results.

At the time of setting up an experiment, samples of the solutions used were taken for estimations of the pH and dissolved oxygen. The former were immediately determined with the use of phenol red, brom thymol blue, brom cresol purple, and methyl red as indicators; the samples for the latter were kept at a constant temperature until Winkler determinations could be made. The results of the Winkler determinations are expressed as cc. of

oxygen per liter. Otherwise, the oxygen content is stated, as is the carbon dioxide content, in terms of percentage saturation. Assuming the applicability of Henry's law to gases in solution, it may be said that when 20 cc. of oxygen-saturated sea water is added to 80 cc. of CO_2-saturated sea water, the resulting solution contains CO_2 at 80 per cent. of saturation value and oxygen at 20 per cent. of saturation value. Since in adding the saturated solutions to the eggs they must experience a small interchange of gases with the air, a solution which was initially free of oxygen is referred to as having a trace of oxygen, while a CO_2-saturated solution is represented as having a CO_2 content of "100 —" per cent. Wherever the tension of CO_2 is expressed in mm. Hg the value given is merely an approximate one, calculated for purposes of comparison with the work of other investigators on the assumption that the tension of carbon dioxide in a saturated solution is 760 mm., minus the vapor pressure of water at the temperature in question.

As already mentioned, the reversibility of the effects of CO_2 was determined by returning the eggs to sea water for development after the desired periods of exposure. Following a rinsing in sea water, the eggs were placed in small Pyrex beakers containing sea water to a depth of about 1.5 cm. Samples of the eggs were removed from the beakers at various times and were preserved for subsequent observation by the addition of a weak solution of formalin in sea water. The fixed eggs were placed in a large hanging drop where, if free from debris, they tended to settle in rows, which simplified the task of determining the percentages of eggs which had undergone the first cleavage. These values, found from time to time after a given exposure, give, when plotted against the minutes after fertilization, a curve which will be referred to as the cleavage curve for that particular exposure. The characteristic S-shape of this curve is related to the variability of the eggs themselves in the manner discussed by Loeb and Northrop (1917) and by Brooks (1918). The time required for cleavage in 50 per cent. of the eggs has in these experiments been used as the most convenient criterion of the cleavage rate, but other percentages could equally well be compared; the time in question can,

of course, be readily found by interpolation from the cleavage curve.

Results.

Before discussing the typical effects of carbon dioxide, it is necessary to rule out the possibility that oxygen lack might be a contributing factor in the results produced, since the method used in saturating the sea water with carbon dioxide causes at the same time a removal of oxygen. Although a complete lack of oxygen has been shown to stop the cleavage process in sea urchin eggs (E. B. Harvey, 1927), the present work indicates that even with an extensive reduction in oxygen tension cleavage is able to continue—and at a rate but little slower than normal. The results of the thirty minute exposures to low tensions of this gas, representing but 14 per cent. to 18 per cent. of those available for the controls, are given in Table I. and show that the cleavage time under these conditions was delayed but a few minutes.

Table I.

The Effect upon Cleavage of Thirty Minute Exposures to Low Oxygen Tensions.

Solution.	cc. of Oxygen per Liter.	Minutes Required for 50 per cent. Cleavage.
Sea water	5.6	60.5
Nitrogen-saturated sea water	0.76	68.5
Sea water	5.48	62.5
Nitrogen-saturated sea water	0.97	64.2

A direct comparison of the effects of low oxygen tension and of high carbon dioxide tension has been made in another experiment, in which each of four portions of egg suspension was exposed for thirty minutes to one of the following solutions:

	pH.	cc. of Oxygen per Liter.
1. Sea water	—	5.15
2. Sea water + oxygen + CO_2 (60% saturated)	5.3	5.56
3. Sea water + nitrogen + CO_2 (60% saturated)	5.3	1.26
4. Sea water, saturated with nitrogen	—	0.55

It will be evident that solutions 3 and 4 were low in oxygen as compared with solutions 1 and 2, while solutions 2 and 3 were high

in CO_2 as compared with solutions 1 and 4. The first cleavage appeared as shown in Fig. 1, where it will be seen that the shortest cleavage time occurred with the sea water control, represented by Curve 1. A lowering of the oxygen tension to slightly over one tenth of the normal value retarded the cleavage time but 4 minutes

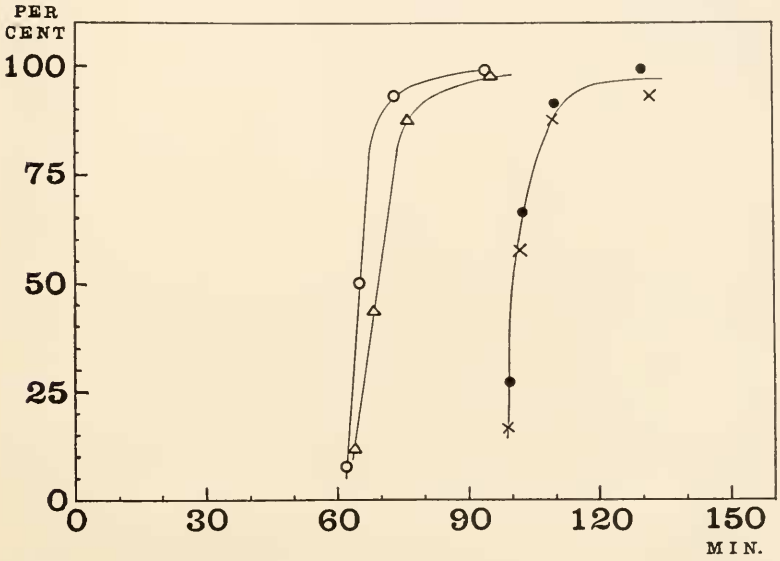

FIG. 1. The relative effectiveness of low oxygen tensions and high CO_2 tensions in delaying the first cleavage of *Arbacia* eggs. Exposures were of thirty minutes' duration. Temperature, 19.8°–20.2°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

	Symbol.	cc. Oxygen. per Liter.	CO_2.
Curve 1	○ Circles	5.15	
Curve 2	× Crosses	5.56	60%
Curce 3	● Dots	1.26	60%
Curve 4	△ Triangles	0.55	

(Curve 4). On the other hand, an increase of the CO_2 content to give pH of 5.3 was sufficiently great to retard the cleavage time 36 minutes, irrespective of the oxygen present, since a single curve serves to represent cleavage in the two solutions which, although showing more than a 4:1 difference in oxygen content, had the same carbon dioxide content. Since from such experiments as

the foregoing the cleavage process is seen to be but little affected by oxygen deficiency over a wide range, the more striking effects observed with CO_2, about to be described in more detail, may justly be attributed to an action of carbon dioxide in which incidental oxygen lack plays little or no part.

Such being the case, the solutions used in the subsequent experiments were simply mixed in a large graduated cylinder and immediately added to the eggs. Siphons were not used nor were Winkler determinations made. Speed in setting up the experiment was important, and in cases where a considerable number of bottles were to be filled, this method had the advantage of expediency. Where the sea water used was saturated with CO_2 (i.e. "100 —" per cent. CO_2, with a trace of oxygen) it seems probable that sufficient oxygen must have entered the solution from the air to prevent any retardation of cleavage from oxygen lack, since a 4:1 mixture of CO_2-saturated and oxygen-saturated sea water (*i.e.* 80 per cent. CO_2 and 20 per cent. oxygen) gave practically the same result. In fact, the results following "100 —" per cent. CO_2 were very similar to those of various tensions down to as low as about 30 or 40 per cent. CO_2. Inasmuch as most of the work here reported is not concerned with more than a semi-quantitative estimate of the gases, it is believed that the method is sufficiently accurate.

In striking contrast to the extensive diminution in available oxygen which the eggs seem to tolerate is the effect upon cleavage of even very small amounts of carbon dioxide. The repression of cleavage which occurs when, in the laboratory, the eggs are subjected to overcrowding is a familiar example of the effects which may be produced simply by the CO_2 which arises from the metabolism of the cells themselves. Experimentally, sea water containing as little CO_2 as 10 per cent. of saturation value was found, after an exposure of twenty minutes to delay cleavage twelve and one half minutes, while exposures of equal length to 30 per cent. and 40 per cent. CO_2 were found to delay cleavage a longer time—about twenty-three minutes. Apparently this latter value of 40 per cent. saturation or 300 mm. Hg represents practically a complete suppression of cleavage since it is approximately the same value as is obtained with higher tensions.

TABLE II.

PERCENTAGE OF EGGS CLEAVED DURING EXPOSURE TO CO_2.

Experiment.	No. 130.	No. 131.	No. 129.	No. 120.
Temperature	22.3°	21.7°–22.1°	21.3°–22.4°	21.2°–21.6°
Per cent. CO_2	15%	20%	30%	80%
pH	*6.25*	*6.2*	*5.05*	*5.2*
Exposures (in minutes):				
80′	10.9%	0%		
90′	27.7%			
100′		1%	0%	
120′			0%	
140′	56.8%			
160′			0%	
200′			6.8%	
300′			43.8%	
640′				0%

It is evident from Table II. that with less than 30 per cent. saturation the repression of cleavage is not complete, but only partial. At a value of 30 per cent., repression is almost complete, since only 7 per cent. and 44 per cent. of the eggs were able to cleave in three and one third and five hours respectively. It will be shown later in the paper that the effects of 30 per cent. and 40 per cent. CO_2 are so similar to those produced by greater amounts, that it is reasonable to suppose that at a saturation of 40 per cent. and over, representing a tension of 300 mm. Hg or more, the suppression of cleavage is practically complete. Therefore not only can very small amounts of CO_2 in the surrounding medium cause an appreciable delay in cleavage, but also its maximum action in suppressing cleavage is approached at values far below saturation.

Notwithstanding the prompt and extensive check upon cleavage which carbon dioxide produces, the reversibility of its effects upon returning the eggs to sea water serves to indicate that the action has been of a narcotic rather than of a purely toxic nature. After exposures of twenty minutes to sea water practically saturated with CO_2 there are usually no abnormalities in cleavage, and larvæ develop which show little or no difference from the normal controls. Following longer exposures, abnormalities appear, although they are relatively few in number with exposures of less than an hour. In spite of the presence of many abnormally

cleaved eggs, a few ciliated larvæ have been found to develop after an exposure of two and one half hours, and the first cleavage has made its appearance in 95 per cent. to 100 per cent. of the eggs subjected to very nearly 100 per cent. CO_2 for this length of time, though relatively few normal larvæ were obtainable after such long exposures. In one experiment—that illustrated in Table III.—exposure of the eggs to 80 per cent. CO_2 for over ten hours still permitted reversibility of the cleavage process to the extent that, six hours after the eggs had been returned to sea water, 88 per cent. were found to have divided, although development was very abnormal and went no farther than the first few cleavage stages. Apparently the process of nuclear division is an extremely powerful one and, as has been observed by others, can persist even when the cell itself is unable to divide.

Fig. 2 is a typical illustration of the series of cleavage curves which are obtained with various lengths of exposure to a relatively high tension of carbon dioxide. It will be observed that the first cleavage ultimately occurs in practically all of the eggs, even after prolonged exposures. The relation of the exposure time to the total retardation is a matter of some importance in indicating the nature of the observed effects. If the narcosis produced by carbon dioxide were complete and were followed by instantaneous recovery the resulting retardation of cleavage, as compared with normal controls, should be exactly equal to the time of exposure. Incomplete narcosis, on the other hand, would tend to shorten, and a more gradual recovery, to lengthen, the period of retardation. Theoretically, a combination of these two effects might conceivably, under a given set of conditions, result in a net retardation exactly equal to the period of the exposure, but it is believed that such a balancing of effects could not account for the results about to be described. Since with all tensions above approximately 30 per cent. saturation the relation of time of exposure to total retardation is essentially the same, it is difficult to imagine that the CO_2 tension should, above this point, either be without effect on a measurable rate of development or that a decrease in this rate should always be followed by a correspondingly increased rate of recovery. The reasonable interpretation of the

facts is that development is practically suppressed by the higher tensions of CO_2. The correctness of this view is also indicated by the fact that cleavage was not obtained during prolonged exposures to such solutions.

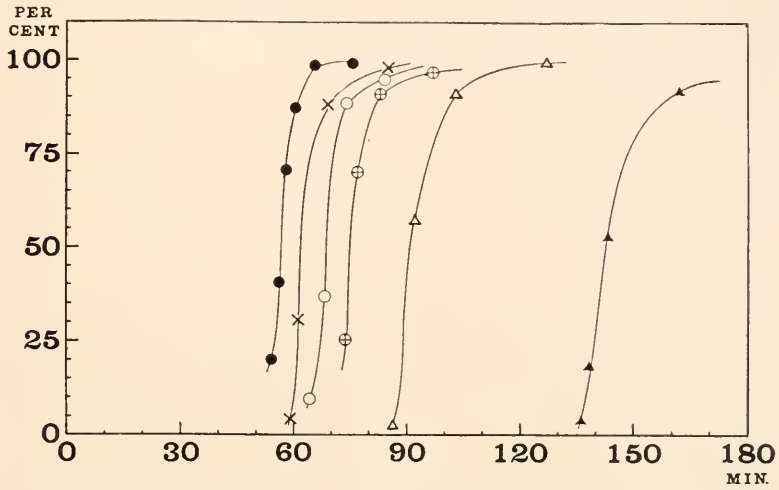

FIG. 2. The times required for the appearance of the first cleavage of *Arbacia* eggs following various lengths of exposure to sea water 80 per cent. saturated with CO_2. Temperature, 21.2°–21.6°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

Symbol.	Length of Exposure.
● Dots	0 minutes
× Crosses	5 minutes
○ Circles	10 minutes
⊕ Crosses in circles	20 minutes
△ Hollow Triangles	35 (?) minutes
▲ Solid Triangles	80 minutes

For each of the six curves the final value, omitted from lack of space, was 100 per cent., except in the case of the twenty minutes exposure, where it was 98 per cent. There have also been omitted the cleavage curves following exposures of 160, 320, and 640 minutes, which ultimately showed cleavage in 97 per cent., 99 per cent., and 88 per cent. of the eggs respectively.

Table III. gives the delay in 50 per cent. cleavage corresponding to each cleavage curve of the complete series.

The relation between time of exposure and retardation of development is shown for a typical experiment in Table III., the values for 50 per cent. cleavage having been taken from the curves in Fig. 2.

Table III.

The Delay in 50 per cent. Cleavage Caused by Various Exposures to 80 per cent. CO_2.

(Experiment illustrated in Fig. 2.)

Exposure Time (in Minutes).	Delay in 50 per cent. Cleavage (in Minutes).
0	—
5′	5′
10′	12′
20′	18′
35′ (?)	34′
80′	86′
160′	186′
320′	370′
640′	750′

It will be observed that with the shorter exposures the recovery of the cleavage process may occur with great rapidity, the delay being but little more than the time corresponding to the period of exposure. Consequently, it has very often been found possible, in performing experiments, to predict the beginning of cleavage with a fair degree of accuracy simply by adding the exposure time to that required for the beginning of cleavage in the normal. As exposures become longer, the greater discrepancy between the exposure time and the delay of cleavage is wholly in one direction —that of prolonging the cleavage time. A further analysis of the data shows even in this respect a simple relation which may best be brought out by plotting the time required for 50 per cent. cleavage against the time of exposure, as has been done in Fig. 3. It will be noticed that straight lines may be drawn through the points representing any given experiment. Those indicated in the figure have been calculated by the method of least squares, with the result that the slopes of all the lines are approximately equal. All may be represented fairly accurately by the equation

$$y = a + 1.18x,$$

where a = the cleavage time of the eggs in the absence of CO_2; x = the time of exposure, and y = the cleavage time of the exposed eggs. Further data are given in Table IV.

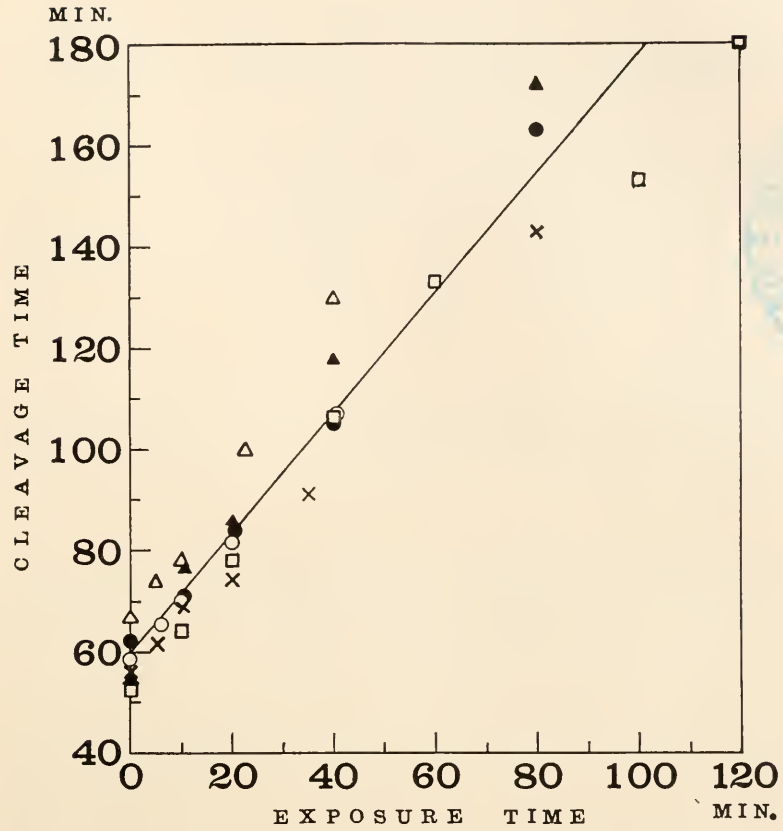

FIG. 3. The relation between the time required for 50 per cent. cleavage of *Arbacia* eggs and the duration of exposure to CO_2 at values from 30 per cent. saturation to practically complete saturation. Abscissa = minutes of exposure to CO_2. Ordinate = minutes required for cleavage in 50 per cent. of the eggs. Details are given in Table IV.

Symbol.	Experiment.
○ Circles	No. 119
× Crosses	No. 120
△ Hollow Triangles	No. 121
▲ Solid Triangles	No. 122
● Dots	No. 128
□ Squares	No. 129

(From lack of space, several values for exposures of over 120 minutes have not been included. These values were, however, used in calculating the equations the average of which—$y = 60.2 + 1.18x$—gives the straight line shown in this figure.) The slope of the curve would be unity, if recovery of the eggs were instantaneous upon their removal from CO_2.

Table IV.

Equations for the Retardation of 50 per cent. Cleavage in *Arbacia* Eggs in Sea Water 30 per cent. to "100 — " per cent. Saturated with CO_2.

Experiment.	CO_2 Content.	Oxygen Content.	Temperature.	Intercept.	Slope.
No. 119.....	"100 —"%	Trace	20.6°–21.2°	58.3	1.192
No. 120.....	80%	20%	21.2°–21.6°	52.6	1.182
No. 121.....	"100 —"%	Trace	20.4°–20.7°	71.	1.191
No. 122.....	"100 —"%	Trace	20.7°–20.8°	65.3	1.194
No. 128.....	40%	20%	20.8°–21.3°	59.35	1.23
No. 129.....	30%	20%	21.3°–22.4°	54.66	1.07
Mean Values.				60.2	1.18

(A preliminary experiment which showed a slope of 1.7 has been omitted from the average since this value differs widely from those subsequently obtained.)

The numerical value—1.18—of the slope of the lines in Fig. 3 shows that the retardation in cleavage produced by a given exposure is nearly, though not exactly, equal to the time of exposure. A value of unity would indicate exact equality. The fact that the slopes of the various lines are nearly the same is an indication of the general similarity, already mentioned, of the CO_2 effects at all tensions above 30 per cent. of saturation. The different values of the intercepts are without significance in this connection, since they represent merely the normal time of cleavage in those eggs which were not exposed to carbon dioxide.

Summary.

1. The first cleavage of the fertilized eggs of *Arbacia* is entirely suppressed, or practically so, in the presence of amounts of carbon dioxide greater than those corresponding to a 40 per cent. saturation of sea water or a tension of approximately 300 mm. Hg. In the presence of smaller amounts of carbon dioxide cleavage is possible, but is greatly delayed.

2. Since a very considerable oxygen deficiency causes only a slight delay in the cleavage process, the factor of oxygen lack is probably a negligible one in the results here described.

3. The effects of a complete suppression of the cleavage process

in sea water practically saturated with carbon dioxide are readily reversible up to exposures of twenty minutes. Beyond that point abnormalities may appear, though after exposures of two and one half hours 95 to 100 per cent. of the eggs ultimately divide.

4. The after effects of exposures of moderate length to carbon dioxide are comparatively slight, the delay in the first cleavage being only a little greater than the actual time of exposure. Mathematically, the relation

$$y = a + 1.18x$$

(where a = normal cleavage time; x = time of exposure; and y = cleavage time of the exposed eggs) has been found to describe fairly accurately the results obtained at 21.4° (± 1.°) with sea water from 30 per cent. saturation to almost complete saturation.

I am glad to have this opportunity to express my gratitude to Dr. M. H. Jacobs for his suggestion of this problem and for his continued interest in the progress of the work.

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